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Comparative Studies on Gametophyte Morphology and Development of Seven Species of Cyatheaceae

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ABSTRACT.—Gametophyte morphology and development of seven species of Cyatheacae in China are described. The spores of the seven species are of typical shape (trilete, tetrahedral) and they exhibit *Cyathea*-type germination. The gametophytes undergo *Adiantum*-type development with occasional *Drynaria*-type development. Filaments are usually 2–3 cells long. The normal adult prothalli are cordate and thalloid with prominent cushions in the middle of the two wings. Prothalli are usually bisexual and antheridia form earlier than archegonia. Lingulate, strap-like and branched prothalli easily grow on the crowded improved Knop's agar media, which produce notches late and produce more antheridia. In distilled water, filamentous prothalli only produce antheridia. The shapes of the mature prothalli of *Sphaeropteris brunoniana* and *Alsophila austro-yunnanensis* are distinct among seven species. Multicellular chlorophyllous hairs appear on dorsal or ventral surfaces in the archegonial region near the notch when the prothallus matures, and the hairs are scaly when they get old. Hairs of the prothallus are like those on the juvenile sporophyte fronds. Vegetative proliferations of old protahlli have been observed.

KEY WORDS.—Cyatheaceae, Gametophyte, Morphology, Development

Gametophyte morphology of ferns including types of spore germination, early development, mature form, trichomes, and gametangia, has been considered to be the significant, defining characteristic of fern taxa (Atkinson and Stokey, 1964; Nayar and Kaur, 1971; Atkinson, 1973). The comparative morphology of the fern gametophyte can be of service in understanding different phyletic groups (Bower, 1923–1928; Stokey, 1951). The details of gametophyte biology are imperfectly known in a large number of species, and the vegetative characters are unreliable when prothalli grow in crowded and unfavorable culture conditions (Atkinson and Stokey, 1964). Therefore, further

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TABLE 1. Cyatheacae species studied and their collection sources. All vouchers are at KUN

Species	Vouchers	Locality, Altitude and Time
Sphaeropteris brunoniana (Hook.) R.M.Tryon	X.Cheng101	Xiaola highway, Mengla,Yunnan 1050 m 2005.8
Alsophila spinulosa (Wall.ex Hook.) R.M.Tyon	X.Cheng103	Botanical Garden of Kunming Institute, Kunming 1600 m 2005. 8
A. costularis Baker	X.Cheng102	A de bo, Jinping, Yunnan 1650 m 2005. 8
A. latebrosa Wall.ex Hook.	X.Cheng106	Xiao wei shan, Hekou, Yunnnan 900 m 2005. 8
A. gigantea Wall.ex Hook.	X.Cheng100	Xiaola highway, Mengla,Yunnan 1050 m 2005. 8
A. austro-yunnanensis S.G. Lu	X.cheng108	Between Pingbian and Hekou, 3 km to Hekou, Yunnan 1460 m 2005.8
A. khasyana T.Moore ex Kuhn	X.cheng109	Between Pingbian and Hekou, 3 km to Hekou, Yunnan 1460 m 2005.8

studies should be performed to understand the development of fern gametophytes.

Cyatheaceae is a family of terrestrial ferns with tree-like trunks and scales (Large, 2004). Studies of Cyatheaceae gametophytes have been performed by some authors (Momose, 1967; Conant, 1990; Khare and Chandra, 1995; Huang *et al.*, 2000; Huang *et al.*, 2001; Wang *et al.*, 2007), however, additional gametophyte morphology and development data are needed and comparisons of gametophyte characters among Cyatheaceae species should be performed; the optimal growing media for the Cyatheaceae gametophytes should be investigated. These data can provide baseline information to inform phylogenetics or the ecology of the species studied.

The average spore sizes of seven Cyatheaceae species, the morphology and development of gametophytes cultured in three different media have been examined in the present study. These findings will add to our understanding of Cyatheaceae gametophytes and their development.

MATERIALS AND METHODS

The species studied and the collection and voucher information are presented in Table 1. In China, the genera of Cyatheaceae are treated in two ways (Ching, 1978; Xia, 1989). The treatment of Xia (1989) is followed in the present study. Voucher specimens are deposited in the Herbarium of Kunming Institute of Botany, Chinese Academy of Sciences (KUN). Spores were obtained from fertile fronds of sporophytes. Pinnae were left to dry at room temperature in paper envelopes to facilitate the opening of the sporangia and expulsion of the spores. Spores were separated from fragments of leaves and sporangia and stored in a refrigerator at about 4°C.

Spores were sown in three different media: improved Knop's agar medium (Liu *et al.*, 1991), a soil medium (Wang *et al.*, 2007) and distilled water. The pH of the first two media types was 5.5–6.5. Before sowing, the spores were sterilized with 4% sodium hypochlorite for five minutes then rinsed with sterilized water four times; between rinses spores were centrifuged at 3500 rpm five times (AD-72 centrifuge).

All cultures were kept in the lab under a 14 hr light /10 hr dark photoperiod provided by artificial light (pink fluorescent illumination) at 1000–1500 Lux. The temperature with light was 22–28°C and 14–18°C in dark.

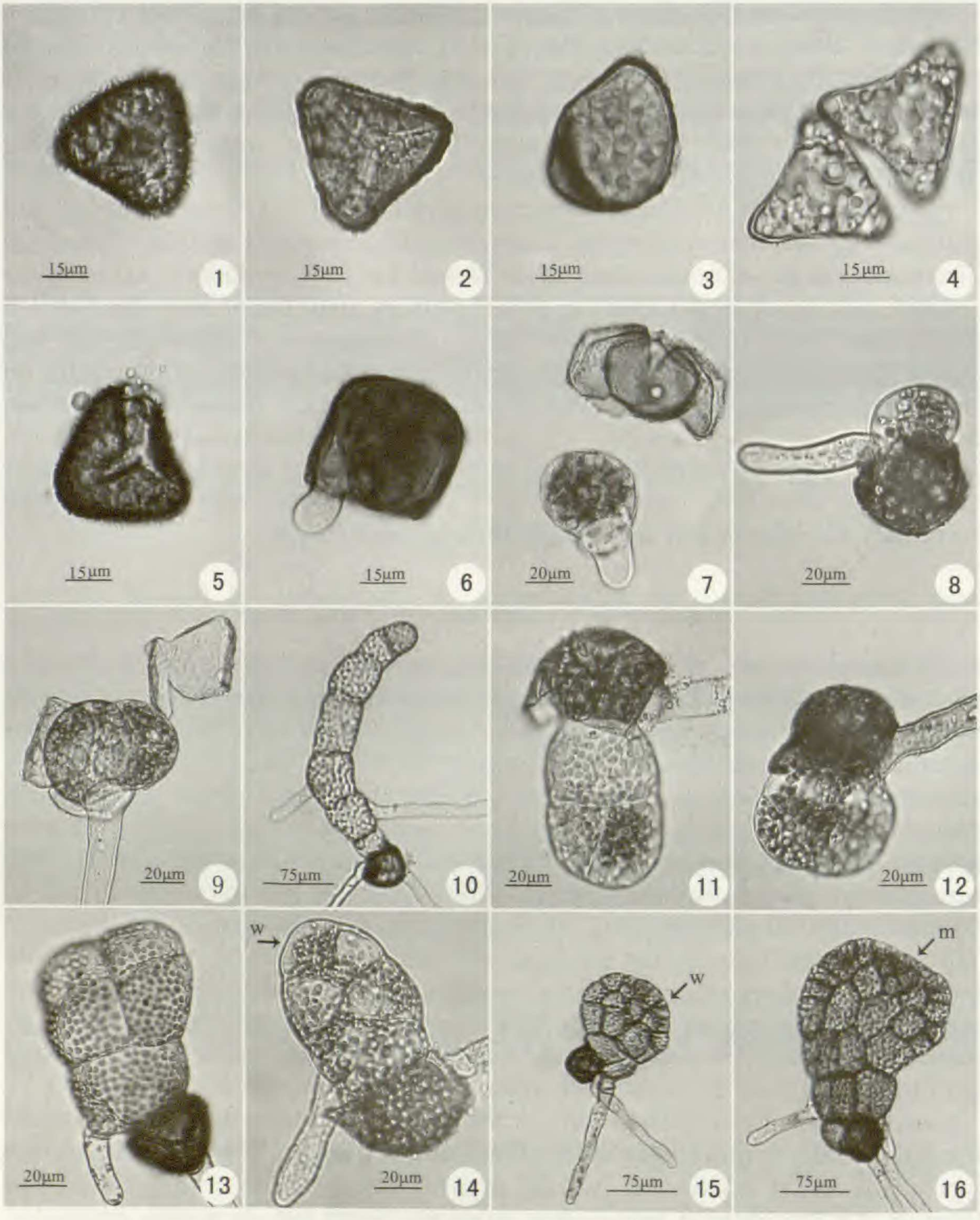
Light microscopes (OLYMPUS BX51) and an anatomical lens (OLYMPUS SZ61) were used to make morphological observations of gametophytes and young sporophytes. For observations, gametophytes and young sporophytes on the solid media were removed from culture and mounted in water. Morphological characteristics of fresh gametophytes were recorded via photomicrography. Thirty spores of each species were measured under light microscope after they were rinsed with distilled water, and the sizes were recorded. The sperm was dyed with Noland tinct liquid.

Results

All spores are typical trilete, tetrahedral, and brown to dark brown. Viewed from a polar perspective, the outlines of the spores are triangular, usually with vertical or concave sides and rounded angles; viewed from the equatorial perspective the outlines are hemispheric or flabellate. The spores of *A. austro-yunnanensis* (Fig. 1), *A. gigantea* and *A. latebrosa* have dense ornamentum and those of *A. costularis, A. khasyana and A. spinulosa* (Figs. 2, 3) have sparse ornamentation and the spores of *S. brunoniana* are rarely seen ornamented under the microscope. The immature spores were found to contain many oil globubes (Fig. 4). Spore sizes are presented in Table 2.

Spores in the Knop's agar medium and distilled water began to germinate between 6–12 days after they were sown, and in the soil media, the spores were found to germinate 3–4 days later than those in the improved Knop's agar media and distilled water (Table 3). A small number of spores of each species germinated 35 days or more, even several months, after sowing.

Germination of the spores was *Cyathea*-type. When the spores germinated the spore walls ruptured at the triradiate ridges (Fig. 5). The first rhizoid was formed by the first division parallel or near parallel to the spore polar axis (Figs. 6, 7); the rhizoid initial was hyaline, 12–15 μm in width, elongated rapidly and had little evident cytoplasm (Fig. 8). The first cell division of the spore also gave rise to the original prothallial cell which contained numerous small oil globules. The second division was perpendicular to the polar axis; by transverse divisions, the 2–3 cell long, uniseriate filament (Fig. 9) was formed in the two solid media; while emerged in the distilled water, the filaments were usually 3–8 cells long (Fig. 10), and sometimes elongated to more than 10 cells long. The filament phase of the gametophyte was usually 5–6 days on the solid media; emerged in distilled water, it could last nearly two months.



FIGS. 1–16. Trilete spores, germination and filamentous phases, cell divisions in the second dimension of filaments and cell plates of Cyathaceae. 1–4. Spores. 1. *A. austro-yunnanensis*. 2. *A. khasyana*. 3. *A. spinulosa*. 4. *S. brunoniana*. 5–6. Germination. 5. *A. gigantea*. 6. *A. costularis*. 7. The first cell division of *A. costularis*. 8. Filament and its rhizoid of *A. khasyana*. 9. Filament and spore's second division of *A.austro-yunnanensis*. 10. Filaments emerged in distilled water of *A. khasyana*. 11–12. First cell divisions in the second dimension. 11. *A. latebrosa*. 12. *A. austro-yunnanensis*. 13. Cell plate without wedge-shaped meristematic cell of *A. austro-yunnanensis*. 14. Cell plate with wedge-shaped meristematic cell of *A. costularis*. 15. Young plate phases with w of *A. khasyana*. 16. Spathulate plate with m of *A. spinulosa*. w = wedge-shaped meristematic cell, m=meristematic zone.

TABLE 2. The spore sizes of Cyatheacae species studied.

Species	Polar axis length	Equatorial axis length
Sphaeropteris brunoniana	25.0–35.0 (29.1) μm	32.5–40.0 (37.7) μm
Alsophila spinulosa	25.0–32.5 (29.3) μm	35.0–40.0 (37.5) μm
A. costularis	25.0–32.5 (29.5) μm	32.5–40.0 (37.8) μm
A. latebrosa	25.0–37.5 (31.3) μm	32.5–42.5 (37.2) μm
A. gigantea	30.0–35.0 (32.2) μm	35.0–42.5 (37.1) μm
A. austro-yunnanensis	30.0–37.5 (34.1) μm	37.5–42.5 (39.5 μm
A. khasyana	30.0–37.5 (33.8) μm	37.5–42.5 (40.2) μm

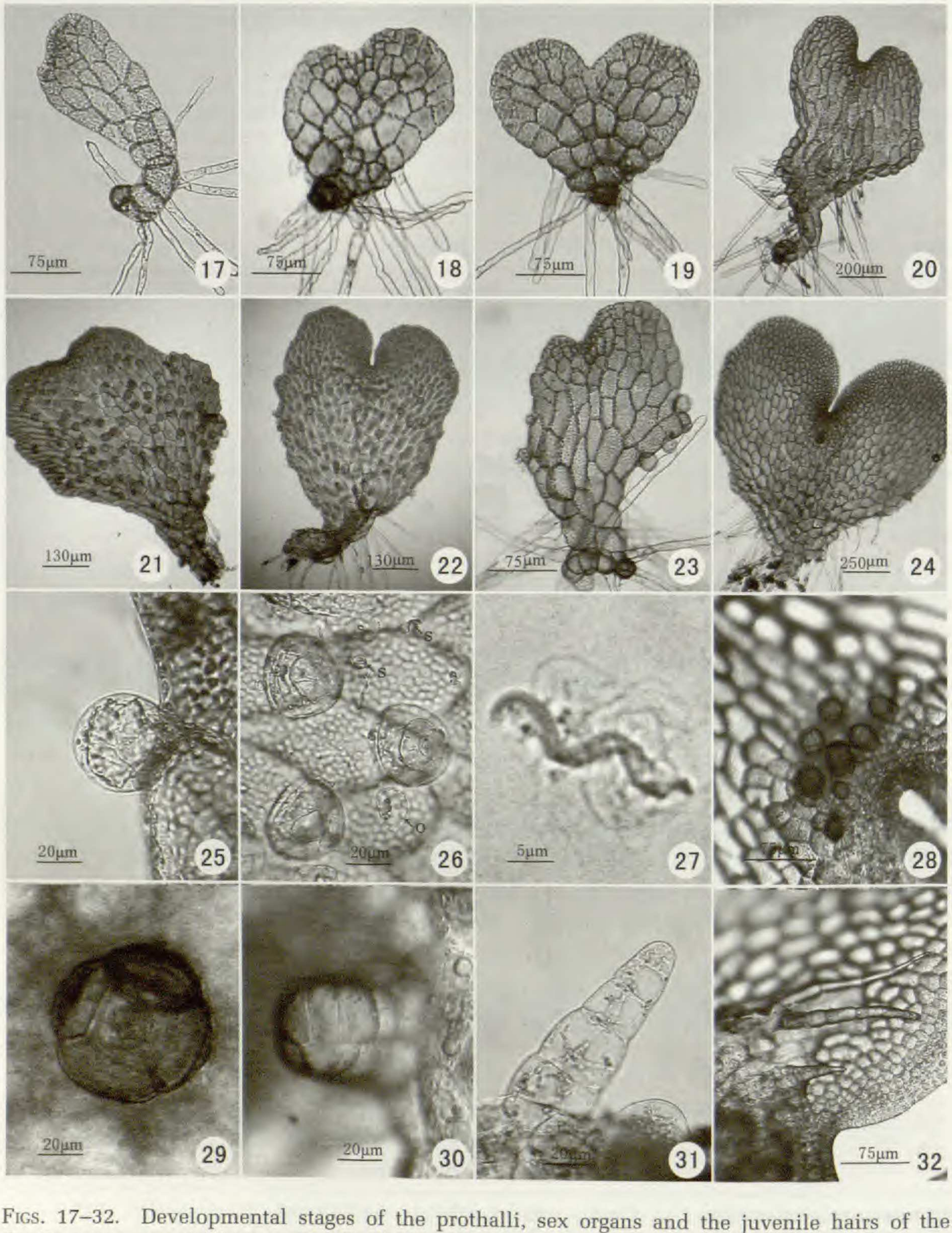
Gametophytes exhibited *Adiantum*-type development, with occasional *Drynaria*-type development, especially in *A. gigantea* and *A. austro-yunnanensis*. Variations in cell division sequence and development rate were observed among species. The apical or subapical cell divided in the second dimension when the filament was 3 cells long, 13–16 days after the spore was sown (Fig. 11). Sometimes, the second filamentous cell began to divide in the second dimension (Fig. 12), which was mostly found in *A. gigantea* and *A. austro-yunnanensis*, occasionally found in *A. spinulosa A. khasyana, A. costularis, A. latebrosa*, and rarely found in *S. brunoniana*.

The spathulate plates began to form in two ways: 1) the initial second dimensional division did not form a wedge-shaped meristematic cell, but only produced a cell plate (Fig. 13); 2) a wedge-shaped meristematic cell formed during the filament stage (Fig. 14). When the cell plate was 3–5 cells wide (Fig. 15), the meristematic cell underwent repeated oblique divisions until it was replaced by a pluricellular meristem, whose activity formed an apical notch (Fig. 16, 17). The wings of the prothallus were sometimes found asymmetric (Fig. 18), but they become symmetric gradually (Fig. 19). The wings were one cell thick and became more curved and ruffled with age (Fig. 20). The pluricellular meristems usually began to form between 17–

TABLE 3. Times of Cyatheaceae spore germination, prothallus meristematic zone appearance and sporophyte appearance

Species	Spore germination time (day)			Apical notch of prothallus appearance time (day) on IKAM	The first sporophyte appearance time (day) on SM
	I KAM	DW	SM		
Sphaeropteris brunoniana	7	6	10	20	175
Alsophila spinulosa	8	7	11	17	118
A. costularis	9	11	14	25	96
A. latebrosa	10	10	13	27	120
A. gigantea	10	9	13	30	204
A.austro-yunnanensis	11	12	15	33	235
A. khasyana	8	7	11	22	110

Note: IKAM= Improved Knop's agar medium DW=Distilled water SM=Soil Medium MZ=Meristematic zone



FIGS. 17–32. Developmental stages of the prothalli, sex organs and the juvenile hairs of the Cyatheaceae gametophytes. 17. Spathulate plate of *A. khasyana*. 18. Asymmetric cordate prothallus of *A. pinulosa*. 19. Symmetric cordate prothallus of *A. spinulosa*. 20. Cordate protallus of *S. brunoniana*. 21–24. Sexual prothalli. 21. *A. kahasyana*. 22. *A. gigantea* 23. *A. kahasyana*. 24. *A. spinulosa*. 25-26. Antheridia of *A. costularis*. 25. Side view of antheridium. 26. Top view of antheridium. 27. Sperm of *A. latebrosa*. 28-30. Archegonia of *A. costularis*. 28. Archegonia group. 29. Top view of archegonium. 30. Side view of the archegonium. 31–32. Prothallial juvenile hairs of *A. costularis*. s=sperm, o= opercular cell.

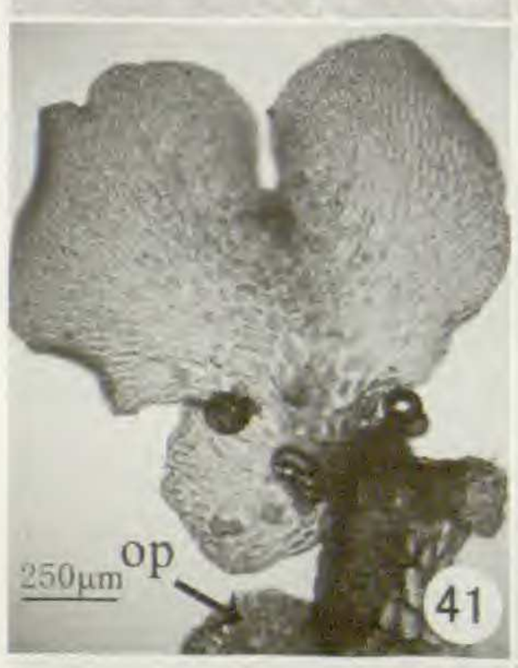
33 days. Among these seven species, *A. spinulosa* and *S. brunoniana* formed the apical notches earlier than the others and *A. austro-yunnanensis* and *A. costularis* were last to form the apical notches (Table 3).

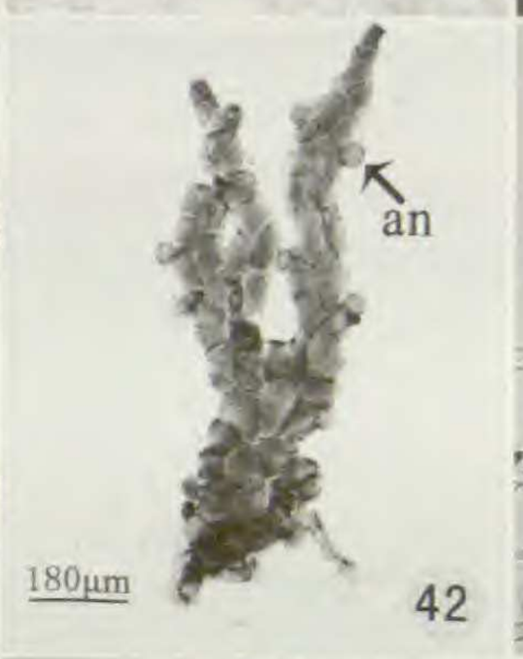
Secondary rhizoids grew from the rhizoid initials or the basal cells of the filaments while the primary rhizoids elongated (Figs. 13, 14). The number of the rhizoids increased with the development of the prothallus. Rhizoids usually formed on the ventral surface of the prothallus, but sometimes they were found on the dorsal surface of the cushion or the wing margins. On the soil medium the rhizoids were usually restricted to the ventral surface of the lower part of the cordate prothalli.

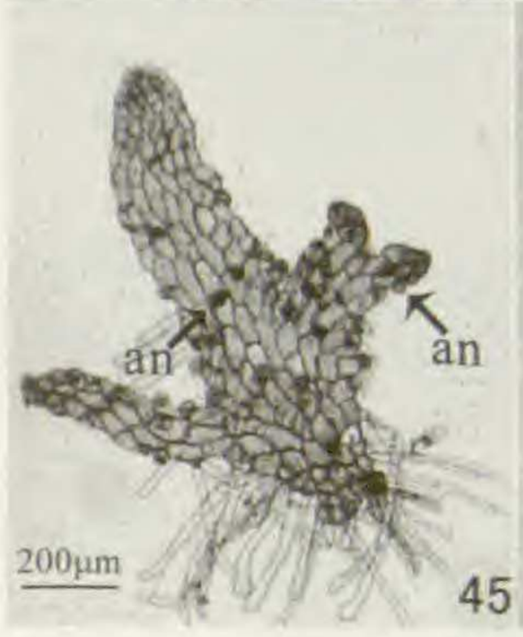
All prothalli produced antheridia earlier than archegonia and the appearance time of archegonia varied corresponding to soil medium and Knop's agar medium. Prothalli began to produce antheridia 30–40 days after spores were sown during late spathulate or primary heart-shaped stages (Figs. 21, 22). Typically, antheridia appeared on the ventral surface of the wings, however some of them were found distributed over the dorsal surface or along the wing margins (Fig. 23). The archegonia were accompanied with cushion appearance on the ventral surface of the prothalli (Fig. 24), 35–50 days after the spores were sown on the soil media, and 60–70 days on improved Knop's agar media. The mature antheridia were about 40.2–50.5 μm in top-view diameter and 30.0–40.3 μm in side-view length (Fig. 25) on the Knop's agar medium, and those on the soil were about 50.5–55.3 μm and 35.5–45.2 μm. The wall of the antheridium was composed of 5 cells: a basal cell, 2 ring cells, a crescent-shaped cell and an elliptical opercular cell. Once the mature antheridium was watered the opercular cells were shed to release spermatozoids (Fig. 26), and 5–6 minutes later the spermatozoids began to develop into sperm. The free-swimming sperm were spiral in form and moved with jerky movement by means of cilia (Fig. 27). The prothalli continued to produce antheridia until they were fertile or dead. The archegonia formed in a group near the notches of the prothalli (Fig. 28); on the soil medium they were about 43.8–54.4 μm in width from the top view (Fig. 29), however they were smaller on the improved Knop's agar medium. The necks of the archegonia were composed of four tiers of cells and were 4–6 cells in length (Fig. 30).

The prothalli on the soil medium usually produced more sex organs than those on the agar media. The prothalli were bisexual although the antheridia and archegonia occurred on each prothallus asynchronously. The cushions grew thicker and longer with increased age, and the notches widened as the cushions grew.

Hairs appeared on the dorsal and ventral surfaces of the cushions in the archegonial regions as or after archegonia had formed. They contained chloroplasts, began as long, uniseriate, spike-like structures (Figs. 31, 32), and grew by intercalary divisions into bi- or tri-seriate, lanceolate structures; then they became multiseriate or broadened into scaly structures with a uniseriate tip; if the fertilizations had been delayed the scaly hairs would have grown to 10–20 cells wide (Fig. 33). Primarily, the hairs on the ventral surface were more abundant than those on the dorsal surface, and then they all became







FIGS. 33–48. Scaly hairs, adult prothalli, juvenile sporophyte, vegetative proliferations of old prothalli and abnormal prothalli of Cyatheaceae. 33. Prothallial scaly hair of *A. latebrosa*. 34–37. Adult prothalli. 34. *A. gigantea*. 35. *A. costularis*. 36. *S. brunoniana*. 37. *A. austro-yunnanensis*. 38. Juvenile sporophyte in *A. khasyana*. 39. Hairs on juvenile sporophyte of *A. costularis*. 40-41. Vegetative proliferation of *S. brunoniana* old prothallus. 42. Falimentous proliferations from *A. kahasyana* old prothallus. 43–48. Abnormal prothalli. 43. Lingulate prothallus of *A. latebrosa*. 44. Strap-like prothallus in *A. kahasyana*. 45. Branched prothallus in *A. spinulosa*. 46. Prothallus with swollen rhizoids of *A. kahasyana*. 47. Prothallus with a mass of rhizoids in *A. latebrosa*. 48. Prothallus emerged in distilled water of *A. spinulosa*. n=notch, an=antheridium, sr=swollen rhizoid, op=old prothallus.

increasingly abundant with increasing prothallus age. The trichomes of *S. brunoniana* occurred and became scaly hairs one month earlier than the others. All hairs did not perish until the prothalli had languished.

The shapes of the mature cordate prothalli were not all the same. In the more crowded soil medium the cordate prothalli elongated and the cushions prolonged with age (Fig. 34); the sex organs increased in density with the elongation of prothallial cushions. If the spores are sown sparsely the cordate prothalli grow wider (Fig. 35). The margins of the wings become more or less curved when the prothalli mature. Among these the wings of *S. brunoniana* curved earlier and the prothalli were butterfly-shaped with extremely flexuous wing margins (Fig. 36). The notches of the *A. austro-yunnanensis* were deep with overlapping or proximate wings above the notches, and the margins of the wings were usually smooth (Fig. 37). The prothalli of the other five species shared the same shape: long or wide cordate with slightly curved wings and slightly flexuous margins.

Mature prothalli began to give birth to sporophytes several months after the spores were sown (Fig. 38), and the approximate time for sporophyte production for each species is presented in Table 3. The long cordate prothalli produce sporophytes later than the wide cordate ones because of the later appearance of the archegonia. On the juvenile sporophyte, muticellular hairs, much like the juvenile hairs on the prothalli, were found on the young fronds (Fig. 39).

Vegetative proliferations of old prothalli, which did not bear sporophytes, were observed first in *S. brunoniana*, seven months after sowing spores on the soil. Then the following phenomenon happened continuously and orderly in *A. costularis, A. gigantea, A. spinulosa, A. latebrosa, A. austro-yunnanensis and A. khasyana.* The young branch arose from a single cell, usually on the margin, less frequently on the surface; rhizoids developed at the base of the branch and the growth soon had the appearance of a young prothallus; the young prothallus grew antheridia and soon became a typical cordate prothallus which also produced archegonia like its parent (Figs. 40, 41). At the same time, the branching filamentous proliferations from old prothalli also appeared, which produced antheridia (Fig. 42).

Lingulate, strap-like, and branching prothalli were found on the crowded improved Knop's agar medium and crowded soil medium, whose notches and cushions were delayed or not formed. They bore antheridia on inconsistent places such as along the margins or on both sides of the prothalli (Fig. 43, 44, 45), and archegonia did not appear until the notches and cushions had formed. Some swollen rhizoids were found on the gametophytes cultured on the improved Knop's agar medium (Fig. 46). If the improved Knop's agar medium were contaminated by fungi or bacteria during the initial stage of gametophyte development, their development was abnormal, and they became covered with a mass of rhizoids all over (Fig. 47). Emerged in distilled water, the filaments continued for nearly two months and then developed into 2–3 cells wide prothalli or filaments with only one or two cells divided lengthways, which

produced antheridia throughout their life span but never produced archegonia unless the cordate prothallus formed (Fig. 48).

Discussion

All spores investigated are trilete and tetrahedral. The polar outline is triangular usually with vertical or concave sides and rounded angles. The aperture arms are 3/4 length of the radius of spores, the length of the polar axis is about 20.5–37.5 μm, the equatorial axis is about 32.5–42.0 μm. There is little discrepancy among the sizes of the spores.

Types of spore germination and gametophyte development have been defined by Nayar and Kaur (1971). The spores studied here exhibit *Cyathea*-type spore germination, in which the filament grows along the polar axis and the first rhizoid appears from the equatorial plane. *Cyathea*-type spore germination is a typical characteristic of Cyatheaceae (Nayar and Kaur, 1971). In *Alsophila denticulata* and *Alsophila metteniana* the formation of the first rhizoid does not occur until the filaments are 3–4 cells long (Huang *et al.*, 2001), which is different from those of the seven species in this study. To ascertain whether the delayed rhizoid formation of *A. denticulata* and *A. metteniana* is different from other species of Cyatheaceae, more studies need to be done. Most of the spores germinate during 6–15 days, but a small number of spores germinate one month or more, which indicates that the spores of Cyatheaceae may have the potential of dormancy or afterripening as do the seeds of some spermatophytes.

Gametophytes undergo *Adiantum*-type development, with occasional *Drynaria*-development especially in *A. gigantea* and *A. austro-yunnanensis*. Comparisons among species grown in the same medium indicate that cell number of filaments differs a little; when comparing the same species among different media types, the filaments in the distilled water are much longer than those on the solid medium. Stokey (1951) reported that if the growth conditions are unfavorable either because of inadequate light or space it promoted filamentous growth. In this study, emerged in distilled water, the Cyatheacae spores lacked adequate light and nutrition, and they grew long filaments. It can be inferred that the cell number of the filament depends upon the cultural conditions to some extent.

Rhizoids are nearly the same between species under normal growth conditions. They are hyaline, have little evident cytoplasm but some protoplast, and they do not branch. However, swollen rhizoids were easily found on the improved Knop's agar media during the gametophyte development. Dyer (1979) reported that on media lacking soluble nitrogen, the rhizoids of ferns became swollen. According to our study, the swollen rhizoids appear when growing in the media containing nitrogen, which suggests that lacking nitrogen is not the only factor leading to the abnormity of the rhizoids.

The prothalli are bisexual; antheridia form earlier than archegonia, and the growing conditions can affect the prothallial sexual balance. The antheridia

walls are composed of 5 cells (Atkinson and Stokey, 1964) and the archegonia necks consist of four tiers of cells, which are 4–6 cell long (Momose, 1967). Nayar and Kaur (1971) and Khare and Chandra (1995) found the archegonial neck to be 6–8 cell long. The crowded solid medium grows more male prothalli, and the distilled water only grows male. If nutrition and light is adequate, and the density of the gametophytes is moderate, more bisexual prothalli will appear early; otherwise, archegonia are formed later, usually one month or more after the antheridial prothalli appear. Investigations indicate that the archegonial prothalli are mostly cordate with apical notches while growing on the sparse soil media and improved Knop's agar media; in the distilled water and on the crowded solid medium the archegonia never or are late to form, which affirms that formation of the archegonia requires adequate nutrition and moderate space.

According to our investigations, the prothalli on the improved agar media are rarely found to produce young sporophytes. On the soil media the prothalli began to bear sporophytes after 96-235 days; *A. costularis* needed the shortest time and *A. austro-yunnansis* needed the longest. This indicates that soil media, among the three examined, is more favorable for sexual propagation in many Cyatheaceae species.

The presence of scaly hairs is characteristic of the Cyatheaceae (Momose, 1967). In this study, the scaly hairs of gametophytes began as long, uniseriate, spike-like structures, and then grew into scaly structures, 10–20 cells wide, with a uniseriate tip. Among species the structures of the scaly hairs are not essentially different; the only variations are that the trichomes appear asynchronously among different species and they begin to divide into scaly hairs at different times. Momose (1967) considered the scaly hairs of *Cyathea* to be 4–7 cells long with the basal or sub-basal cell splitting into 2 cells, however, the authors believe, the above hair styles are the juvenile stages of scaly hairs of the Cyatheaceae. In this study, scaly hairs appear on dorsal or ventral surfaces of the cushions near the notches when the prothalli are mature or near maturity, however, Wang (2007) found that trichomes grew all over the prothallus surfaces in *A. costularis*. In our study we only found the trichomes in the archegonial regions of the prothalli, and we did not find trichomes growing all over the surface. We observed that the juvenile trichomes on the prothalli and juvenile fronds were very similar.

Prothalli of most fern taxa are capable of regenerating new prothalli from old ones (Atkinson and Stokey, 1964; Nayar and Kaur, 1971). Vegetative proliferations of the old prothalli are found in this study: young prothalli and branching filamentous proliferations are formed on the old prothalli. The branching filamentous proliferations on old prothalli of Cyatheaeae in the present study bear antheridia, the characteristics of which are like the gemmae investigated by Farrar and Dassler (Farrar, 1967; Dassler and Farrar, 1997; Dassler and Farrar, 2001). Whether or not the structures observed in this study act as gemmae needs further investigation.

The development of Cyatheaceae prothalli responds differently to different media. Typically cordate prothalli are observed on the sparse solid media;

lingulate, strap-like or branched prothalli, which delay or never produce notches, easily grow on the crowded solid media and they only produce antheridia and never produce archegonia until notches appear; filamentous prothalli are usually found in distilled water. However, even given these developmental differences, the normal morphologies of the mature prothalli are comparable. According to our results, the mature prothallus of *S. brunoniana* is margin-curved and butterfly-shaped; those of *A. spinulosa, A. latebrosa, A. costularis, A. gigantea, A. khasyana and A. austro-yunnanensis* are heart-shaped; however, among these, the prothallus of *A. austro-yunnanensis* is different from other species for its notch shape and special wings.

The cultures of the improved Knop's agar media are easily contaminated. Once infected by bacteria or fungi the prothalli will stop developing and instead produce clones to enhance their longevity. When the critical growing conditions are not satisfied, shapes, sexual balance, and sexual function of gametophytes will be affected.

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